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Expression of Proliferation Cell Nuclear Antigen (PCNA) in Peripheral Erythrocytes of Triploid Rainbow Trout *Oncorhynchus mykiss* (Walbaum)

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Abstract: The proliferation of erythroid cells in peripheral blood was detected by anti-PCNA monoclonal antibody (PC10) with a means of immunocytochemistry in diploid and triploid rainbow trout. The rate of PCNA positive erythroid cells in triploidy peripheral blood was detected by flow cytometry. The erythrocytes which were positive for PCNA were confined in the cells with high nuclear-cytoplasmic ratio. About 6.4% erythrocytes were found positive for PCNA in triploid rainbow trout while only 0.4% in diploids. And the PCNA positive rate of the erythrocytes was 0.37% in diploids and 8.39% in triploids in average. PCNA, a proliferating marker is found to be expressed only in immature cells in the peripheral erythrocytes of triploid rainbow trout. This suggests that peripheral erythrocytes with the ability of proliferating are immature cells.

Key words: Rainbow trout, triploid, PCNA, immature erythrocyte, erythroid cells, China

INTRODUCTION

Erythrocytes, developed in hematopoietic organs are commonly distinguished in Peripheral Blood (PB) of rainbow trout (Lane and Tharp, 1980). It has been reported that immature erythrocytes could go directly into PB due to the lower level of cell differentiation (Benfey and Sutterlin, 1984). So, PB was a place not only for erythrocytes exercising their function but also for their development. The proportion of immature erythrocytes in polyploid fish is higher than that of diploids. This is due to the polyploidy which made hematopoietic organs so burdened that many immature erythrocytes entered PB (Benfey, 1999).

Unlike mammal, mature erythrocytes are nucleated in most of the teleost. In some species, especially polyploids, a few peripheral erythrocytes show abnormal morphous which were considered as cytological marker for triploidy (Benfey and Sutterlin, 1984). There is also a question regarding how abnormal erythrocytes come about. It is hypothesized as erythrocytes undergoing cell division (Murad *et al*., 1993; Wang *et al*., 2010) or just a morphological change to increase information exchanges (Liu *et al*., 2003). Based on the knowledge up to today, the available data are still less accurate and based on deduction. Therefore, researchers aim to provide more information in this study.

PCNA plays an important role in DNA replication. So, it has been used in the studies of cell proliferation in paraffin sections just as preadipocytes in rainbow trout and hematopoietic tissues in zebra fish (Bouraoui *et al*., 2008; Leung *et al*., 2005). In this study, the expression of PCNA in peripheral erythrocytes of the triploid rainbow trout was examined to investigate whether peripheral erythrocytes are able to proliferate and which kind of erythrocytes has this ability.

MATERIALS AND METHODS

Twelve diploid and twelve triploid 2 years old fish were obtained from Heilongjiang River Fishery Research Institute. The diploids were 211.38+/-30 g in weight, 25.57+/-3.15 cm long in average. The triploids were 225+/-8.89 g in weight and 25.95+/-2.82 cm long. Triploids were induced by heat shock after fertilization. The ploidity of fish were all determined with flow cytometry for DNA content.

Morphological examination: Blood samples were collected from caudal vein with heparinized syringes and blood smears for light microscope observation were fixed with methanol and stained with Wright's. Selecting 50 field randomly and choosing 50 cells in each field to calculated the proportion of abnormal erythrocytes and immature erythrocytes.

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Western blot analysis of PCNA: Immunoblotting samples were collected from fresh diploid spleen, head kindey and triploid blood and rats spleen is used as control. Crude protein was extracted and electrophoresed through 15% SDS-PAGE gel and transferred to nitrocellulose filter membrane membranes. The membranes were incubated with mouse anti-PCNA monoclonal antibody (diluted 1:100) and then incubated with HRP conjugated goat-anti-mouse IgG. The reaction was detected by ECL plus chemiluminescence.

Immunocytochemical and immunohistochemistrial examination: PCNA expression in the PB and organs of diploid ovary and heart was determined by the method of immunohistochemistry. Smears were incubated with affinity purified anti-human PCNA (mouse monoclonal antibody (clone PC10, eBioscience) 1:100 diluted with PBS) at 4°C overnight after inactivating the activity of endogenous peroxidase andthen incubated with HRP Goat anti-mouse IgG at room temperature for 30 min and detected by DAB. Controls were incubated with PBS instead of anti-PCNA antibodies.

Detection by flow cytometry: PCNA expression level in di and triploid rainbow trout was examined by flow cytometry. Peripheral erythrocytes were separated with lymphocyte separating medium fixed with cold methanol. Cells were washed with PBS twice and re-suspended in PBS incubated with PE labeled PCNA monoclonal antibody for 60 min at 37°C. Unbound antibody was removed by centrifugation and washed with PBS. The negative control was treated with isotype control (PE Mouse IgG2a), instead of PE labeled PCNA monoclonal antibody. Each blood sample had a negative control to count for the nonspecific fluorescence in the samples. Ten thousand cells were collected to detect of the proportion of cells expressing PCNA according to the fluorescence signal. Data were analyzed with SPSS 11.5 and t-test was used for significance.

RESULTS AND DISCUSSION

The morphology of erythrocytes: Compared with that in diploid rainbow trout, the erythrocytes and their nucleus in the triploids were longer and slender (Fig. 1a and b). Immature erythrocytes, characterized by an oval shape, near round nucleus and large nucleus-to-cytoplasm ratio were seen in both diploids and triploids with a percentage of 8.2 and 11.9%. The difference was significant ($p<0.05$). Abnormal erythrocytes were also observed in triploid PB, accounted for 8.5% of all erythrocytes (Fig. 1c and d), diploids showed a very small proportion of erythrocytes which displayed similar nuclear division (about 0.1%).

Western blot analysis of PCNA: The specific crossreactivity of the antibody (PC10) with PCNA in rainbow trout was confirmed by immunoblotting. A 36 kDa band was observed (Fig. 2) in all four samples.

Immunocytochemical and immunohistochemistrial analysis: Brown particle deposition was seen in peripheral erythrocytes of rainbow trout indicating that theses cells were PCNA positive. In both di and triploid rainbow trout, the PCNA signals were seen only in the cells with larger nucleus-to-cytoplasm ratios which were immature erythrocytes (Fig. 3a and b).

In triploids, a percent of 57.9% of the immature erythrocytes were PCNA positive cells. The mature erythrocytes and abnormal erythrocytes did not show PCNA expression (Fig. 3b). In diploids, only 2 PCNA positive cells were found. As controls, heart tissue section did not show PCNA expression (Fig. 3c) and numerous PCNA positive cells were found in ovary

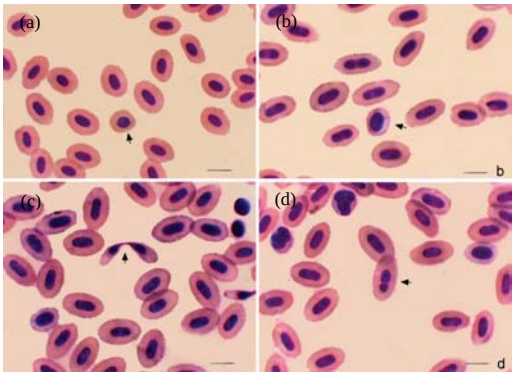

Fig. 1: Photomicrograph of erythrocytes in di and triploid rainbow trout; a) Immature erythrocyte in diploids; b) Immature erythrocyte in tiplids; c and d) Abnormal erythrocytes in triploids. Bar = 10 μm

Fig. 2: Immunoblotting analysis of PCNA protein in rainbow trout. A protein of 35-36 kDa was observed in Rat spleen (R), Liver (L) and Spleen (S) of rainbow trout, Peripheral Blood of triploid rainbow trout (PB)

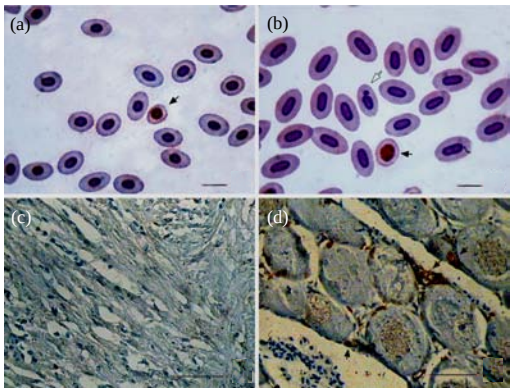

Fig. 3: PCNA immunostaining in erythrocytes of PB; a) Shows PCNA positive cells with the morphous of immature erythrocyte in diploids PB; b) Shows PCNA positive cells with the morphous of immature erythrocyte (black arrow) and abnormal erythrocyte (white arrow) in triploids PB; c) Shows no immunoreactive nuclei was found in heart; d) Shows immunoreactive nuclei found in oogonium of ovary (arrow). Bars: a, b = 10 mm; c, d = 50 mm

sections. The positive staining was observed mostly in oogonia and rarely in oocytes (Fig. 3d). Oogonia undergo a series of mitosis and form the primary oocytes (Babin *et al*., 2007) so, it was strongly positive for PCNA. In differentiated myocardial cells where no cellular proliferation and DNA replication occur, PCNA staining is negative. This illustrates that PCNA could be used as a proliferative marker of cells in rainbow trout.

Flow cytometry detection of erythrocyte: Results of flow cytometry were compared and indicate the difference of PCNA expression level between di and triploids (Fig. 4a and b). PCNA positive cells were 0.4 and 6.4% in diploid and triploid, respectively. The difference was significant ($p<0.01$). The rates of three types of cells in di and triploid rainbow trout were shown in Table 1.

The PCNA test is able to specifically detect cells with DNA replication (Bauer and Burgers, 1990). PCNA is highly conserved in eucaryon and therefore the commercial monoclonal antibody (PC10) could crossreact with distantly related species. In this study, PC10 crossreacted specifically with a of 36-37 kDa polypeptide in rainbow trout tissues and mouse spleen which is consistent with PCNA of other teleost (Morrison *et al*., 2006) and suggested that the PC10 could be used for PCNA detection of rainbow trout. Erythrocytes were generally considered to generate from hemopoietic organ and fully developed in PB.

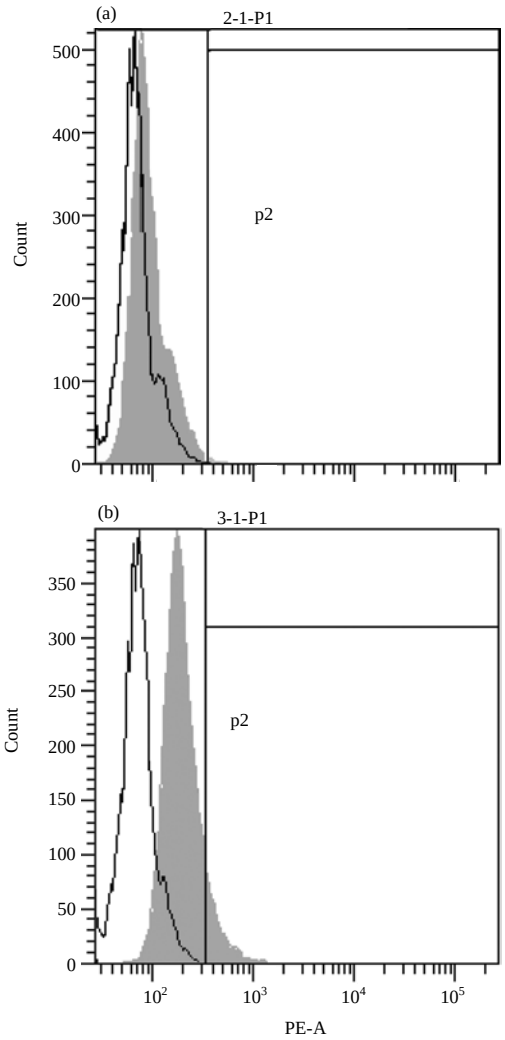

Fig. 4: The difference of PCNA expression level between di and triploids

Table 1: The rates of three types of cells in di- and triploid rainbow trout (%)

Samples	Immature erythrocyte	PCNA positive cell	Abnormal erythrocyte
2n	8.20	0.4	0.1
3n	11.9	6.4	8.5

However, many studies showed that erythrocytes in PB of fish could undergo a further division. If the cells divide, PCNA would express in nuclei during G1 and S phase (Lin *et al*., 1995). In the research neither mature erythrocytes nor abnormal ones showed PCNA expression. Instead, strong immunoreactivity observed in immature erythrocytes showed that they were undergoing a process of cell proliferation which is supported by a similarly positive staining for PCNA in oogonia of ovary and negative in heart tissue. Which means peripheral erythrocytes of rainbow trout do have ability to proliferate but this ability was restricted within immature ones.

In the current study, the number of immature erythrocytes is slightly lower for diploids than percentage for triploids. But the proportion of PCNA positively stained immature erythrocytes was greatly increased in the triploids compared with that of diploids. This results suggest that more than half of the immature erythrocytes in triploids have the ability to multiply and are ready for division. Next, researchers would like to discuss the relationship between immature erythrocytes and abnormal erythrocytes. If cell division occurs in PB then dividing cells should be observable. Consistent with the division hypothesis, abnormal cell was the only cell type that fits the characteristics of cell division. This results are consistent with Wang *et al.* (2010), abnormal cells could be speculated to be the products of cell division being in amitosis. This also, tells us that abnormal cells might be intermediate statuses of dividing immature erythrocytes which is the same as suggested by Murad *et al.* (1993).

Few PCNA expressing erythrocytes and abnormal cells indicated that the diploid immature erythrocytes are also capable of proliferation but the capability was inhibited by certain mechanism. The recovery of proliferation capability in triploids may result from the additional set of chromosomes or stress susceptible.

CONCLUSION

The result of this study, the proportion of immature erythrocytes in fishes may increase due to the elevation of environmental pressure (Lane and Tharp, 1980; Valenzuela *et al.*, 2002) and so do the abnormal erythrocytes (O'Keefe *et al.*, 2001). Triploid is far less tolerant to pressure than diploid (Ojolick *et al.*, 1995). Also, increasing the size of triploid cells will lead to the decreas of erythrocyte count and also reduce oxygen transportation capability. Immature erythrocytes may be a stress response in triploid to increase the number of erythrocytes and compensate for blood oxygen transport capability.

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